Fungi evolved right on track

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Abstract: Dating of fungal divergences with molecular clocks thus far has yielded highly inconsistent results. The origin of fungi was estimated at between 660 million and up to 2.15 billion y ago, and the divergence of the two major lineages of higher fungi, Ascomycota and Basidiomycota, at between 390 million y and up to 1.5 billion y ago. Assuming that these inconsistencies stem from various causes, we reassessed the systematic placement of the most important fungal fossil, *Paleopyrenomycites*, and recalibrated internally unconstrained, published molecular clock trees by applying uniform calibration points. As a result the origin of fungi was re-estimated at between 760 million and 1.06 billion y ago and the origin of the Ascomycota at 500–650 million y ago. These dates are much more consistent than previous estimates, even if based on the same phylogenies and molecular clock trees, and they are also much better in line with the fossil record of fungi and plants and the ecological interdependence between filamentous fungi and land plants. Our results do not provide evidence to suggest the existence of ancient protolichens as an alternative to explain the ecology of early terrestrial fungi in the absence of land plants.

Key words: Ediacaran, Eurotiomycetes, Late Proterozoic, Lecanoromycetes, Pezizomycotina, protolichens, Sordariomycetes, Vendian

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INTRODUCTION

In the past 15 y several studies aimed at dating evolutionary divergences in the fungal tree of life with molecular clock methods, either as primary objective or as a side product (Berbee and Taylor 1993, 2001, 2007; Simon et al 1993; Doolittle et al 1993; Wang et al 1999; Redecker et al 2000; Heckman et al 2001; Douzery et al 2004; Hedges et al 2004; Peterson et al 2004; Padovan et al 2005; Taylor and Berbee 2006). The origin of fungi was estimated at between 660 million and up to 2.15 billion y ago and the divergence of the fungal-animal from the plant lineage at between 780 million and up to 2.5 billion y ago (Taylor and Berbee 2006). The divergence of the two major lineages of higher fungi, Ascomycota and Basidiomycota, was dated at between 390 million y and up to 1.5 billion y ago (Berbee and Taylor 1993, 2007; Taylor and Berbee 2006).

Even considering that some of these studies are experimental, these estimates are highly inconsistent, varying by a factor of 3–5 depending on the node considered and are mostly not in accordance with the fossil record, which suggests that multicellular plants, animals and fungi go back to not more than 650 million y in the Late Proterozoic (Vendian or Ediacaran period). Taylor et al (2004: 188) said, ''This result leaves us to wonder what Fungi were doing on Earth for a billion of years before they were preserved as the fossils we know to exist,'' and Hedges et al (2004: 14) said, ''With so many major lineages of fungi appearing hundreds of millions of years prior to the Phanerozoic, the virtual absence of Precambrian fungal fossils has been surprising.'' The notion that the origin and diversification of fungi predated the evolution of land plants raised speculation that early fungi could have been lichenized (Eriksson 2005), which led Hawksworth (2005) to suggest reconsidering Retallack's (1994, 1995, 2007) idea that the oldest known multicellular fossils from the Vendian or Ediacaran period might represent in part ancient lichens.

While these ideas are intriguing the underlying dating estimates are problematic, as shown by their lack of consistency. Another usually overlooked factor is the phylogenetic interdependence between the fungal, animal and plant lineages. All published phylogenies agree that the fungal-animal split occurred after the split from the plant lineage, which by default makes the plant lineage older than either the fungal or the animal lineage, if only by a short time

(Heckman et al 2001, Hedges et al 2004, Steencamp et al 2006, Moreira et al 2007, Yoon et al 2008). Setting back the origin and diversification of fungi in a molecular clock tree thus automatically sets back the plant lineage as well. In fact studies that estimated the origin of the fungi at more than 1.5 billion y ago also dated the origin of land plants at 1–1.1 billion y ago (Heckman et al 2001, Hedges et al 2004), although the fossil record for land plants does not go back more than 430 million y (Gensel 2008). It is misleading therefore to compare molecular clock estimates of fungal divergences with the fossil record of land plants and speculate about the ecology of ancient fungi, when the same molecular clock estimates require that lands plants must have evolved much earlier than suggested by the fossil record. In other words it makes little sense to accept a molecular estimate that dates fungi back 1.5 billion y and land plants 1.1 billion y but then put fungal divergences in context with the fossil record of land plants dated at 430 million y ago. Coincidentally in all dating studies that include the fungal and plant kingdom with their major lineages resolved, the postulated origin of filamentous fungi is well in line with the postulated origin of land plants, independent of the absolute estimates (Heckman et al 2001, Douzery et al 2004, Hedges et al 2004).

The inconsistencies between molecular clock estimates and the fossil record of multicellular organisms, which begins in the Vendian (Ediacaran) period, together with the nature of these fossils (Knoll et al 2006, Xiao and LaFlamme 2009), suggests that there is a problem with molecular clock estimates instead of a lack of corresponding fossils from the Late Proterozoic that would support such estimates. Even if the fossil record is incomplete, there is a high level of consistency in the fossils known, clearly showing a pattern of evolution of different life forms and repeated mass extinctions. Missing fossils as explanation for discrepancies with molecular clock estimates thus would require a systematic error in the fossil record, such as the absence of complete biota of multicellular organisms that at some time existed but fossilized only much later. For example dating the origin of major lineages of multicellular organisms at about 1–1.5 billion y ago implies a gap in the fossil record of these lineages of 350–850 million y. While this is not impossible it is inconsistent with the fact that the first multicellular, fossilized organisms, the Ediacarans, exhibit morphology very different from extant plant, fungal and animal lineages. If plant, animal and fungal lineages originated much earlier, at that point in time (i.e. 350–850 million y after their presumed origin) one would expect that fossils reflect the morphological disparateness of these lineages instead of a distinct fossil biota such as the Ediacarans. An ancient origin of the fungal lineage also does not imply that fungi in the strict sense were around that early. All dating estimates show that the evolution of filamentous fungi occurred much later than the origin of the fungal lineage itself, suggesting that for a long time after their origin fungi were heterotrophic, unicellular, flagellate, aquatic organisms, not much different from other protists.

The observed inconsistencies in dating fungal divergences could stem from misclassification of the fossil record, incorrect setting of calibration points, use of basal and external calibrations based on substantial extrapolations that impose potentially incorrect substitution rates on the fungal lineage, incorrect phylogenies and inappropriate molecular clock methods. By reassessing the systematic placement of the most important fungal fossil, *Paleopyrenomycites*, and by applying uniform fossil calibration points to the different published molecular clock trees, we show that dating estimates can be made surprisingly consistent, even when retaining the diverse molecular clock trees used in the original studies. The results suggest that fungi evolved and diversified more or less concurrently to the evolution of the major plant lineages and terrestrial ecosystems.

THE FUNGAL FOSSIL RECORD AND PALEOPYRENOMYCITES

Although the fossil record for fungi is meager, the known fossils cover almost all major fungal lineages. Fossil Glomeromycota date from 400–460 million y ago, Basidiomycota clamp connections from 290 million y ago and a variety of fungal remnants from the 400 million y old Lower Devonian Rhynie chert, including the oldest unequivocal euascomycete fossil, *Paleopyrenomycites* (Simon et al 1993; Redecker et al 2000; Taylor et al 1999, 2004, 2005). The latter has been used to calibrate the origin or diversification of the pyrenomycetes or more precisely the Sordariomycetes (Redecker et al 2000, Heckman et al 2001, Peterson et al 2004, Padovan et al 2005, Taylor and Berbee 2006). Its exact systematic position is disputed (Taylor et al 2004, 2005; Eriksson 2005; Padovan et al 2005; Taylor and Berbee 2006; Berbee and Taylor 2007) and it was argued that *Paleopyrenomycites* could belong to any extant group of perithecioid Ascomycota, including the Dothideomycetes and Eurotiomycetes. It was even suggested that it could fall outside the euascomycetes (Pezizomycotina), and possible links with the enigmatic taphrinomycete *Neolecta* (Landvik et al 2003) were discussed (Eriksson 2005, Taylor and Berbee 2006, Berbee and Taylor 2007).

Potential misclassification of *Paleopyrenomycites* stems from its initial publication with four selected

TABLE I. Principal features of *Paleopyrenomycites* based on the fossil reconstruction (Taylor et al 2005) compared to extant major Ascomycota lineages (focusing on lineages producing in part perithecioid ascomata). The fossil agrees in most aspects with either Pezizomycetes or the marine fungus *Orcadia* (of uncertain phylogenetic affinities)

Taxon	Relationship to host	Ascomata	Ontogeny	Hamathecium	Ascus type	Ascospore septation
Taphrinomycetes	Parasitic	—	—	—	Splitting	Nonseptate
Neolectomycetes	Mutualistic?	Neolectoid	—	—	Splitting	Nonseptate
Saccharomycetes	Saprobic Parasitic	—	—	—	Deliquescent	Nonseptate
Paleopyrenomycites	Parasitic	Perithecioid	Ascohymenial	Compact	Operculate	Nonseptate?
Pezizomycetes	Saprobic Parasitic Mutualistic	Apothecioid Perithecioid	Ascohymenial	Compact	Operculate	Nonseptate
Orcadia	Parasitic?	Perithecioid	Ascohymenial	Compact	Operculate	Septate
Leotiomycetes	Variable	Apothecioid Cleistothecioid	Ascohymenial	Compact	Poricidal	Nonseptate
Dothideomycetes	Saprobic	Perithecioid	Ascolocular	Loose, deliquescent	Fissitunicate	Septate
	Parasitic	Apothecioid				
Sordariomycetes	Saprobic Parasitic	Perithecioid	Ascohymenial	Loose	Poricidal	Variable
Lecanoromycetes	Lichens	Apothecioid Perithecioid	Ascohymenial	Compact	Rostrate	Variable

figures suggesting a pyrenomycete (Taylor et al 1999). In the formal description (Taylor et al 2004) the complete set of 45 figures was misprinted, and the paper was republished (Taylor et al 2005). Dating studies before Taylor and Berbee (2006) did not have access to the republished figures and had to rely on the previous classification of the fossil as a pyrenomycete, and this has become well established in the literature. Also, while Taylor et al (2004, 2005) accurately described all features visible in the beautifully preserved fossil, they did not discuss one significant character described and depicted in FIG. 27: the operculate ascus of *Paleopyrenomycites*. The validity of this trait was discussed by Eriksson (2005), who also had not seen the republished figures when putting forward his protolichens hypothesis. As with all fossil evidence it is not impossible that the operculate asci depicted by Taylor et al (2005) are an artifact, but the micrographs are convincing and make an operculate opening mechanism more likely than a poricidal, rostrate or fissitunicate type.

Molecular phylogeny has revolutionized the systematics of Ascomycota, showing that traditional classifications based on fruit body types or ascoma ontogeny do not necessarily reflect phylogenetic relationships (Lindemuth et al 2001; Lutzoni et al 2001, 2004; Grube et al 2004; Lumbsch et al 2004; Wedin et al 2005; James et al 2006; Miadlikowska et al 2006; Spatafora et al 2006; Hibbett et al 2007; Hofstetter et al 2007; Schoch et al 2009). The supported classes have little in common with the traditional separation of Pyrenomycetes and Discomycetes or Ascohymeniales and Loculoascomycetes. Schoch et al (2009) demonstrated that perithecial Ascomycota evolved multiple times from apothecial relatives, the most striking example being the Ostropales, which include closely related apothecial and perithecial lineages (Schmitt et al 2009). On the other hand ascus dehiscence type appears to be a conservative trait, with a progression from splitting and deliquescent forms in the basal Ascomycota to operculate types in the Pezizomycetes (Samuelson 1978a–d) and the odd marine fungus *Orcadia* to the poricidal, fissitunicate and rostrate types found in all remaining classes, now recognized as superclass Leotiomyceta (Schoch et al 2009).

Placement of *Paleopyrenomycites* based on fruit body shape is thus potentially equivocal, whereas its operculate asci would exclude it from the Leotiomyceta and consequently all pyrenomycete lineages (TABLE I). The ascus type places the fossil either in the Pezizomycetes or near *Orcadia*, depending on the interpretation of the ascospores of the fossil as simple or septate. The latter character is ambiguous from the micrographs provided by Taylor et al (2005) because the presumed septa of *Paleopyrenomycites* have a strongly oblique orientation and might represent artifacts of fossilization. *Orcadia* differs from the Pezizomycetes by septate ascospores, but its phylogenetic position is unknown. It also has perithecia, while most Pezizomycetes are characterized by apothecial ascomata. However perithecial and cleistothecial

forms do exist in the Pezizomycetes as well, such as in genus *Orbicula* and the hepaticolous *Octosporella* and relatives (Corner 1929; Döbbeler 1979, 1980, 1997; Hansen et al 2005). Perithecial ascomata in otherwise apothecial lineages evolve through neotenic retention of initially closed ascomata, especially in groups with cleistohymenial ascoma ontogeny (apothecia starting out closed and opening by a widening pore), which includes a large part of the Pezizomycetes (Corner 1929, 1931, 1935; van Brummelen 1967; Pfister 1978, 1993; Grube et al 2004; Hansen and Pfister 2006). The ontogeny of the apothecial pezizomycete *Byssonectria* (Pfister 1993) and the perithecial *Octosporella jungermanniana* (Corner 1929) show stages similar to those depicted for the *Paleopyrenomycites* fossil: ascogonial filaments (Pfister 1993: FIGS. 7–11, Taylor et al 2005: FIGS. 13, 14, 16), periphyses (Corner 1929: FIG. 5, Taylor et al 2005: FIG. 12) and ascoma wall structure (Corner 1929: FIG. 5, Pfister 1993: FIGS. 12, 17, 20, Taylor et al 2005: FIGS. 9, 10, 17).

Other evidence that supports placement of *Paleopyrenomycites* in Pezizomycetes instead of Sordariomycetes is the well defined, compact hymenial layer of comparatively short asci and paraphyses of more or less equal length (Taylor et al 2005: FIGS. 7, 8, 19, 20). Other than in apothecial forms, such hymenia are known from certain ascolocular lineages (Barr 1987), but in those cases ascoma development, hamathecium structure and ascus type deviate strongly from *Paleopyrenomycites*. Ascospores in many pyrenomycetes are actively discharged through the narrow ostiole by individual asci reaching the ostiole at maturity. Taylor et al (2005: 282) assumed a similar mechanism for the fossil, although the ascospores in *Paleopyrenomycites* are not discharged through the ostiole but instead accumulate in the cavity above the hymenium (Taylor et al 2005: FIGS. 7, 8, 11, 19, 20, 44). This is reminiscent of the synchronized ascospore discharge in many Pezizomycetes, in which the ascospores accumulate on and above the hymenial surface.

As a consequence *Paleopyrenomycites* is best placed either in the Pezizomycetes or the Pezizomycotina incertae sedis but most certainly outside and basal to the Leotiomyceta. According to Schoch et al (2009), based on the phylogeny of extant lineages, the ancestral characters for the Pezizomycotina (i.e. filamentous Ascomycota with true ascomata) include saprobic or parasitic relationships with hosts and apothecioid ascomata with ascohymenial ontogeny, whereas the ascus dehiscense type is equivocal. Thus *Paleopyrenomycites* could be placed anywhere within the Pezizomycotina stem lineage or crown or else within the Pezizomycetes stem lineage or crown (FIG. 1).

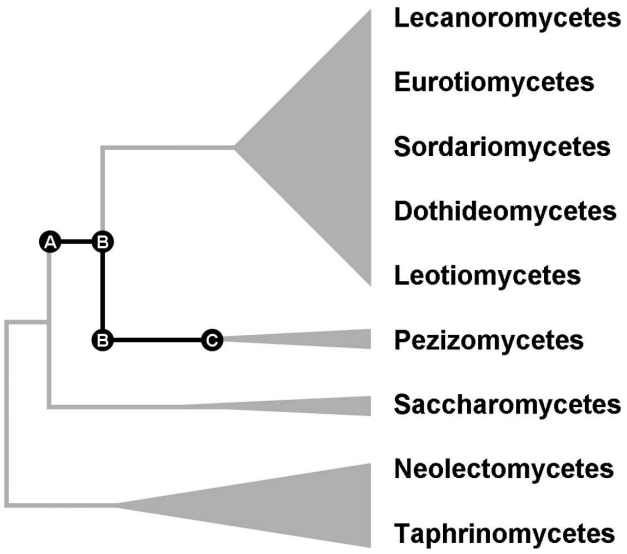

FIG. 1. Most likely placement of the Paleopyrenomycites fossil in the Ascomycota tree: (A) = Pezizomycotina stem base, (B) = Pezizomycotina crown (= Pezizomycetes stem base), (C) = Pezizomycetes crown. Ascomycota phylogeny simplified after Schoch et al (2009).

PROBLEMS IN CALIBRATING FUNGAL MOLECULAR CLOCK TREES

Calibration of molecular clock trees depends on appropriate positioning of fossils as well as careful selection of nonfossil or external calibration points. Only four studies (Berbee et al 1993, 2001; Redecker et al 2000; Peterson et al 2004) exclusively used several fungal fossils to calibrate a fungal molecular clock tree, and two further studies estimated fungal divergence times by exclusively using the *Paleopyrenomycites* fossil (Taylor and Berbee 2006: three out of seven scenarios). Other estimates added or exclusively used external or basal, nonfungal or nonfossil calibration points (Berbee and Taylor 2001, Heckman et al 2001, Douzery et al 2004, Padovan et al 2005, Taylor and Berbee 2006: four out of seven scenarios; Berbee and Taylor 2007).

The basal, nonfossil calibration points were taken from Dolittle et al (1996) and Wang et al (1999), who estimated plant-animal-fungal divergences at between 970 million and 1.6 billion y ago. The use of these estimates as basal calibration points is questionable for at least two reasons. Due to their basal position, they will affect the calibration of the entire tree and reduce the effect of actual fossil calibration, especially if the latter is used to set the minimum age limit of a node only. In Heckman et al (2001) the calibration of the Sordariomycetes (Pyrenomycetes) lineage with the *Paleopyrenomycites* fossil has no effect because the origin of that lineage is exclusively calibrated by the basal tree node and the fossil hence appears midway

within the Sordariomycetes (Pyrenomycetes). Also such basal estimates are derived from extrapolations based on much younger fossil calibration points; Wang et al (1999) used 310 million y old fossils as oldest calibration point and then estimated the age of the basal node of the tree at almost 1.6 billion y ago, thus extrapolating by the factor 5. For this to be correct one would have to assume absence of vertical rate variation below the oldest fossil calibration point (i.e. between the 310 million y old node and the basal node), a questionable assumption given the fact that molecular clock methods now specifically allow for such variations. Extrapolated estimates therefore are unreliable and should not be used as calibration points themselves, especially because this might lead to circular conclusions. Padovan et al (2005) said that their results confirmed those provided by Heckman et al (2001) and Hedges et al (2004), but all three studies actually used the same basal calibration point provided by Wang et al (1999). The same problem applies to the use of external, nonfungal calibration points, such as internal animal or plant divergences, because these might impose potentially incorrect rate variations onto the fungal tree (Taylor and Berbee 2006, Berbee and Taylor 2007). The fact that there are many more animal than plant or fungal species, by several orders of magnitude, suggests that evolutionary rates are not necessarily the same between the major kingdoms.

Even in molecular trees exclusively calibrated with fungal fossils there are problems of the exact calibration point and whether the fossil lineage is actually represented in the tree. In their first tree Taylor and Berbee (2006) used the divergence of Sordariomycetes as calibration for the *Paleopyrenomycites* fossil. Placing the same fossil at the Sordariomycetes origin would have resulted in 1.7 times younger divergence times. In a second scenario *Paleopyrenomycites* represented the Pezizomycotina but no basal taxon of that lineage was included in the tree, resulting in an overestimation of divergence times if the current Ascomycota phylogeny (Schoch et al 2009) is taken into consideration. The same applies to the estimation of Taylor and Berbee (2006) placing the fossil at the Pezizomycotina crown node, which in their molecular clock tree does not include the Pezizomycetes and thus overestimates the divergence time by nearly 10% (TABLE II). Another problem is the use of multiple fossil calibration points. Multiple calibration points do not contribute to the dating estimate unless the calibrated nodes are fixed on both sides. As a consequence fossil calibration of a molecular clock tree ultimately will be determined by the fossil that pushes back the nodes further than any other fossil. In the fungal lineage it appears that this is achieved by the 400 million y old *Paleopyrenomycites* fossil, while septate hyphae from 438 million y ago and the Glomalean fossil from 460 million y ago result in slightly younger estimates (Redecker et al 2000, Peterson et al 2004).

GRAPHICAL RECALIBRATION OF INTERNALLY UNCONSTRAINED TREES

The published estimates for the origin and divergence of the fungal tree show standard deviations of 34–55% depending on the node, and the relative minimum–maximum range varies between 117% and 151% of the mean (TABLE II). We wanted to know whether this uncertainty was due to the different phylogenetic or molecular clock methodology or merely a result of inconsistent or incorrect node calibration. Vertical rate variation below and above fixed calibration points result in relative compression or expansion of portions of a molecular clock tree; therefore the tree or parts of it cannot simply be recalibrated by graphical stretching or condensing it, unless the lineage of interest is internally unconstrained, that is calibrated by a single (internal or external) node only. Of the 15 molecular clock trees reviewed here six had no internal constraint in the fungal lineage that would cause internal vertical rate variation (Berbee and Taylor 2001, Heckman et al 2001, Padovan et al 2005: first scenario; Taylor and Berbee 2006: three out of seven scenarios), and two further trees had no internal constraint above the main internal calibration point (Padovan et al 2005: second scenario, Taylor and Berbee 2006: second illustrated tree). The seven internally unconstrained trees encompass the entire range of variation of published dating estimates and hence suffice for this recalibration exercise.

Molecular clock trees in which the fungal lineage had been calibrated at a single node in the original study (no other external or internal constraints) were graphically aligned by setting the Ascomycota-Basidiomycota origin to 1 and present time set to 0. All major nodes were recalculated as distances relative to one (with present time set to 0). For all realigned trees the *Paleopyrenomycites* fossil, with an estimated age of 400 million y, was placed at three different calibration points (TABLE II): (i) Sordariomycetes origin (stem base), (ii) Pezizomycotina divergence (crown) and (iii) Pezizomycotina origin (stem base; corresponding to the Pezizomycotina-Saccharomycotina divergence). In two cases in which the Pezizomycotina divergence node was not available (because no Pezizomycetes were included in the molecular clock tree), we applied a correction of 1.07 relative to the Sordariomycetes origin node, based on the most

recent phylogeny of the Ascomycota (Schoch et al 2009). For the study by Berbee and Taylor (2007), in which no internal Ascomycota node was available, we applied a correction of 0.64 relative to the Ascomycota origin node to determine the Pezizomycotina divergence node also based on Schoch et al (2009).

The resulting estimates based on recalibration were subjected to a nested trend convergence analysis, assuming that phylogenies and molecular clock methods improve over time; for any given node the two values representing the recalibrations of the first two published estimates were averaged and the resulting value was averaged with the next recalibrated estimate and so on, resulting in a new series of n – 1 values. This iteration was repeated with the resulting series until converging to a single value, which was considered the trend value of the series. Trend values were obtained for all nodes for two different calibrations: *Paleopyrenomycites* positioned at the Pezizomycotina divergence (crown) node and the Pezizomycotina origin (stem base) node. Because the *Paleopyrenomycites* fossil must have appeared subsequent to the origin of the lineage that it represents the range provided by the Pezizomycotina divergence and origin node appears to be the best estimate (FIG. 1 between points A and B).

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As a result of the recalibration (TABLE II) standard deviation of node estimates decreased to 3–20% and minimum-maximum range variation decreased to 7–60% of the mean (TABLE III). Placing the fossil in the Sordariomycetes, as suggested by previous studies but with a corrected node assignment, resulted in up to 27% decrease of divergence time estimates depending on the node; placing it at the Pezizomycotina divergence node resulted in up to 31% decrease. The most dramatic changes were observed when placing the fossil at the origin of the Pezizomycotina, with up to 47% lower divergence times. For example the origin of the fungi was re-estimated at 720 ± 140 million y ago, as compared to 1230 ± 430 million y ago in the original publications (TABLE III).

Restricting the calibration to a fossil and its corrected placement in the molecular clock tree substantially affected the results, whereas the underlying phylogeny or molecular clock method only marginally influenced the results, as shown by the reduced overall variation of estimates (TABLE III). For instance Berbee and Taylor (2001) assumed a globally constant rate and used a phylogeny inconsistent with current knowledge, with the Sordariomycetes sister of the remaining Pezizomycotina; yet our recalibration of their tree resulted in estimates close to the overall recalibrated estimates. We also found that studies which exclusively used several fungal fossils appropriately positioned in the tree (Redecker et al 2000, Berbee and Taylor 2001, Peterson et al 2004) came closest to our recalibrated estimates (TABLE II).

The recalibrated estimates for the origin and divergence of the fungal tree of life are in line with the fossil record of the plant, animal and fungal kingdoms, especially if *Paleopyrenomycites* is conservatively placed at the base of the Pezizomycotina (TABLE IV). It appears that the major fungal lineages evolved and diversified in line with the evolution and diversification of vascular plants and terrestrial ecosystems from limnic and marine macro-algae and early nonvascular land plants. This is especially so because the divergence estimates for the fungal kingdom go hand in hand with the divergence estimates for the major plant lineages (FIG. 2). Our most conservative estimate places the diversification of early chytrids and zygomycetes at around 630 million y ago, parallel to the diversification of limnic macro-algae. The Glomeromycota appeared 600 million y ago, which allows for the well preserved 460 million y old fossils (Simon et al 1993). The Ascomycota and Basidiomycota originated around 500 million y ago, more or less parallel to the first appearance of primitive land plant fossils. The diversification of the higher ascomycetes, the Pezizomycotina, estimated at around 320 million y ago, correlates well with the diversification of vascular plants as documented by the fossil record. The main classes of Pezizomycotina, here represented by Sordariomycetes, Eurotiomycetes and Lecanoromycetes, evolved and diversified parallel to the evolution and diversification of early terrestrial ecosystems around 270–290 million y ago in the Carboniferous and Early Permian.

Our estimate dates the divergence of the plant-animal-fungal lineages at 820–1200 million y ago. This is at the lower end of other published estimates (Doolittle et al 1996, Taylor and Berbee 2006) and 25–50% lower than the most ancient estimates at 1400–1600 million y ago (Wang et al 1999, Heckman et al 2001, Hedges et al 2004, Yoon et al 2004, Berbee and Taylor 2007, Zimmer et al 2007). While our recalibration exercise suggests the split of the plant, animal and fungal lineages to have occurred much more recent than indicated by these studies, we have to reiterate that extrapolations that go beyond two times the age of the oldest fossil used for calibration, in this case 400 million y, should be calculated with care. Our recalibrated estimate of the plant-animal-fungal split therefore is not reliable.

Our recalibrated estimates do not provide evidence to suggest the existence of ancient protolichens, as

TABLE II. Original divergence estimates for the fungal tree of life and recalibrated estimates using three different positions for the *Paleopyrenomycites* fossil (Sordariomycetes stem base, Pezizomycotina crown, Pezizomycotina stem base). PLAN = plant lineage, ANIM = animal lineage, FUNG = fungal lineage (Fungi), GLOM = Glomeromycota, ASCO = Ascomycota, PEZI = Pezizomycotina, SORD = Sordariomycetes, EURO = Eurotiomycetes, LECA = Lecanoromycetes. Original = original divergence estimates, SORD base = Sordariomycetes stem base, PEZI crown = Pezizomycotina crown, PEZI base = Pezizomycotina stem base (recalibrated estimates given only when original tree was internally unconstrained in the fungal lineage and graphical recalibration was possible). (A) and (B) in Padovan et al (2005) and Taylor and Berbee (2006) refer to different original calibrations which resulted in different recalibrated estimates; (1), (2) and (3) in Taylor and Berbee (2006) refer to different original positioning of the *Paleopyrenomycites* fossil: (1) = Sordariomycetes crown, (2) = Pezizomycotina crown, (3) = Ascomycota crown

		Nodes									
Source	Calibration	PLAN-ANIM-FUNG	FUNG base	FUNG crown	GLOM base	ASCO base	PEZI base	PEZI crown	SORD base	EURO base	LECA base
Berbee and Taylor 1993: fungal fossils (constant rate)											
	Original	—	—	560	490	390	310	280	280	280	—
	SORD base	—	—	780	690	560	440	400	400	400	—
	PEZI crown	—	—	780	690	560	440	400	400	400	—
	PEZI base	—	—	710	630	510	400	360	360	360	—
Simon et al 1993: basal divergence 1000 mya (Wolfe et al 1989), monocot diverg. 200 mya (Knoll 1992)											
	Original	—	—	—	410	—	—	—	—	—	—
Dolittle et al 1996: extrapolated from animal divergences 550–100 mya											
	Original	1000	970	—	—	—	—	—	—	—	—
Wang et al 1999: extrapolated from animal divergences 310–110 mya											
	Original	1580	1500	—	—	—	—	—	—	—	—
Redecker et al 2000: fungal fossils 460–90 mya											
	Original	—	850	630	600	590	470	400	380	400	—
Berbee and Taylor 2001: basal diverg. 970 mya (Dolittle et al 1996), fung. fossils 460–40 mya (const. rate)											
	Original	—	880	820	590	550	360	320	320	250	250
	SORD base	—	1100	1020	730	680	450	400	400	310	310
	PEZI crown	—	1100	1020	730	680	450	400	400	310	310
	PEZI base	—	980	910	650	600	400	360	360	280	280
Heckman et al 2001: basal divergence 1580 mya (Wang et al 1999)											
	Original	1580	1580	1470	1320	1170	1090	730	680	680	—
	SORD base	930	930	880	790	700	650	430	400	400	—
	PEZI crown	860	860	810	730	650	600	400	370	370	—
	PEZI base	570	570	540	490	430	400	270	250	250	—
Hedges et al 2004: extrapolated from bird-mammal divergence 310 mya											
	Original	1610	1510	1000	—	1000	980	590	550	550	—
Padovan et al 2005 (A): basal divergence 1580 mya (Wang et al 1999)											
	Original	—	—	1420	—	1210	1070	970	890	750	850
	SORD base	—	—	640	—	540	480	430	400	340	380
	PEZI crown	—	—	590	—	500	440	400	370	310	350
	PEZI base	—	—	540	—	450	400	360	340	280	320

TABLE II. Continued

Source	Calibration	Nodes									
		PLAN-ANIM-FUNG	FUNG base	FUNG crown	GLOM base	ASCO base	PEZI base	PEZI crown	SORD base	EURO base	LECA base
Padovan et al 2005 (B): basal divergence 970 mya (Dolittle et al 1996), *Paleopyrenomycites* fossil											
	Original	—	—	890	—	790	660	570	520	430	480
	SORD base	—	—	—	—	—	—	430	400	340	370
	PEZI crown	—	—	—	—	—	—	400	370	310	340
	PEZI base	—	—	—	—	—	—	310	290	240	260
Taylor and Berbee 2006 (A): *Paleopyrenomycites* fossil											
	Original (1)	2570	2170	1630	—	1490	1190	770	720	720	—
	Original (2)	1450	1230	920	—	840	—	430	—	—	—
	Original (3)	780	660	490	—	450	350	230	210	210	—
	SORD base	1420	1210	910	—	820	660	420	400	400	—
	PEZI crown	1350	1150	860	—	780	630	400	380	380	—
	PEZI base	860	730	550	—	500	400	250	240	240	—
Taylor and Berbee 2006 (B): external divergences mosquito 235 mya and monocots 206 mya											
	Original	1210	1030	790	—	730	600	430	400	400	—
	SORD base	—	—	—	—	—	—	430	400	400	—
	PEZI crown	—	—	—	—	—	—	400	370	370	—
	PEZI base	—	—	—	—	—	—	310	290	290	—
Berbee and Taylor 2007: external divergence animals 700 mya (Douzery et al 2004), monocots 170 mya (Magallon and Sanderson 2005), Glomeromycota fossil 460 mya											
	Original	1370	1150	770	500	420	—	270	—	—	—
	SORD base	—	—	—	—	680	—	430	—	—	—
	PEZI crown	—	—	—	—	630	—	400	—	—	—
	PEZI base	—	—	—	—	490	—	310	—	—	—

TABLE III. Mean values and standard deviation (in mya) plus range and percentage variation of original and recalibrated divergence times for selected nodes of the fungal lineage. Bold indicates nodes recalibrated with *Paleopyrenomycites*. PLAN = plant lineage, ANIM = animal lineage, FUNG = fungal lineage (Fungi), GLOM = Glomeromycota, ASCO = Ascomycota, PEZI = Pezizomycotina, SORD = Sordariomycetes, EURO = Eurotiomycetes, LECA = Lecanoromycetes

Node	Observations	Mean ± SD	Range and percentage variation relative to mean
Original publications			
PLAN-ANIM-FUNG	9	1460 ± 34%	780–2570 (123%)
FUNG base	11	1230 ± 35%	660–2170 (123%)
FUNG crown	12	950 ± 39%	490–1630 (120%)
GLOM base	6	650 ± 52%	410–1320 (140%)
ASCO base	13	790 ± 43%	390–1490 (139%)
PEZI base	11	690 ± 48%	310–1190 (128%)
PEZI crown	13	490 ± 45%	230–970 (151%)
SORD base	11	490 ± 42%	210–890 (139%)
EURO base	11	460 ± 42%	210–750 (117%)
LECA base	4	480 ± 55%	250–850 (125%)
Recalibrated at Sordariomycetes stem base			
PLAN-ANIM-FUNG	4	1300 ± 19%	930–1420 (38%)
FUNG base	5	1130 ± 11%	930–1210 (25%)
FUNG crown	7	860 ± 14%	640–1020 (44%)
GLOM base	3	740 ± 7%	690–790 (14%)
ASCO base	8	700 ± 15%	540–820 (40%)
PEZI base	7	570 ± 18%	440–670 (40%)
PEZI crown	10	420 ± 3%	400–430 (7%)
SORD base	9	**400**	—
EURO base	9	370 ± 9%	310–400 (24%)
LECA base	3	350 ± 9%	310–380 (20%)
Recalibrated at Pezizomycotina crown			
PLAN-ANIM-FUNG	4	1240 ± 20%	860–1380 (42%)
FUNG base	5	1090 ± 12%	860–1170 (28%)
FUNG crown	7	830 ± 16%	590–1020 (52%)
GLOM base	3	720 ± 3%	690–730 (6%)
ASCO base	8	670 ± 15%	500–800 (45%)
PEZI base	7	540 ± 17%	440–650 (39%)
PEZI crown	10	**400**	—
SORD base	9	380 ± 3%	370–400 (8%)
EURO base	9	350 ± 11%	310–400 (26%)
LECA base	3	330 ± 6%	310–350 (12%)
Recalibrated at Pezizomycotina stem base			
PLAN-ANIM-FUNG	4	790 ± 18%	570–860 (37%)
FUNG base	5	750 ± 20%	570–980 (55%)
FUNG crown	7	620 ± 23%	540–910 (60%)
GLOM base	3	590 ± 15%	490–650 (27%)
ASCO base	8	500 ± 9%	430–600 (34%)
PEZI base	7	**400**	—
PEZI crown	10	300 ± 15%	250–360 (37%)
SORD base	9	290 ± 17%	240–360 (41%)
EURO base	9	270 ± 14%	240–360 (44%)
LECA base	3	280 ± 11%	250–320 (25%)

postulated by Eriksson (2005), to explain an origin of fungi long before the evolution and diversification of land plants. It is conceivable that ancient saprotrophic fungi lived on dead algal material belonging to Charophyta or Ulvobionta, which include several lineages of terrestrial green algae, especially in periodically dry, limnic ecosystems, and the occurrence of mutualistic fungal-algal associations at that time cannot be excluded. However naming such hypothetical associations ''protolichens'' is misleading because they cannot be considered precursors to modern lichens, which evolved from nonlichenized

TABLE IV. Range of recalibrated divergence times (trend values) of the fungal lineage based on placement of *Paleopyrenomycites* at the Pezizomycotina crown and stem base, using a nested trend convergence analysis. PLAN = plant lineage, ANIM = animal lineage, FUNG = fungal lineage (Fungi), GLOM = Glomeromycota, ASCO = Ascomycota, PEZI = Pezizomycotina, SORD = Sordariomycetes, EURO = Eurotiomycetes, LECA = Lecanoromycetes

Node	Range of trend values based on recalibration	Geological time period
PLAN-ANIM-FUNG	≈ 820–1200 mya	Middle to Late Proterozoic
FUNG base	≈ 710–1060 mya	Middle to Late Proterozoic
FUNG crown	≈ 630–820 mya	Late Proterozoic
GLOM base	≈ 600–720 mya	Late Proterozoic
ASCO base	≈ 500–650 mya	Late Proterozoic to Cambrian
PEZI base	≈ 400–520 mya	Late Cambrian to Late Ordovician
PEZI crown	≈ 320–400 mya	Late Devonian to Early Carboniferous
SORD base	≈ 290–380 mya	Middle Devonian to Late Carboniferous
EURO base	≈ 270–350 mya	Early Carboniferous to Early Permian
LECA base	≈ 280–330 mya	Early Carboniferous to Early Permian

ancestors (Schoch et al 2009) and, according to our estimates, much later and after the evolution of vascular plants. It is not necessary to assume mutualistic associations between fungi and algae to explain the evolution of ascomata; all lower Pezizomycotina as well as the taphrinomycete *Neolecta* have ascomata but are nonlichenized (Schoch et al 2009), and throughout the Ascomycota and Basidiomycota many saprotrophic and parasitic lineages have well developed fruit bodies. The ascomata of the oldest euascomycete fossil, *Paleopyrenomycites*, are immersed in vascular plant tissue, suggesting that they evolved by forming a protective wall layer between the fungal hymenium and the host tissue. In the absence of other evidence it is conceivable that this represents an ancient type of ascomata, with no need to postulate a lichen-like precursor.

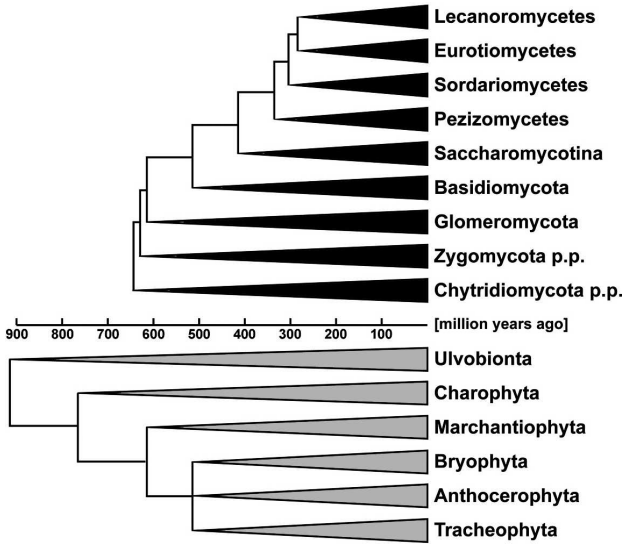

FIG. 2. The recalibrated fungal tree of life compared to the evolution of green plants, based on our most conservative estimates (TABLE IV). Green plant phylogeny and dating combined and simplified from various sources (Wang et al 1999; Heckman et al 2001; Hedges et al 2004; Yoon et al 2004, 2008; Steencamp et al 2006; Berbee and Taylor 2007; Moreira et al 2007: Zimmer et al 2007).

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